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Immune-related mechanisms participating in resistance and susceptibility to glutamate toxicity

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Abstract

Glutamate is an essential neurotransmitter in the CNS. However, at abnormally high concentrations it becomes cytotoxic. Recent studies in our laboratory showed that glutamate evokes T cell-mediated protective mechanisms. The aim of the present study was to examine the nature of the glutamate receptors and signalling pathways that participate in immune protection against glutamate toxicity. We show, using the mouse visual system, that glutamate-induced toxicity is strain dependent, not only with respect to the amount of neuronal loss it causes, but also in the pathways it activates. In strains that are genetically endowed with the ability to manifest a T cell-dependent neuroprotective response to glutamate insult, neuronal losses due to glutamate toxicity were relatively small, and treatment with NMDA-receptor antagonist worsened the outcome of exposure to glutamate. In contrast, in mice devoid of T cell-dependent endogenous protection, NMDA receptor antagonist reduced the glutamate-induced neuronal loss. In all strains, blockage of the AMPA/KA receptor was beneficial. Pharmacological (with α_2-adrenoceptor agonist) or molecular intervention (using either mice overexpressing Bcl-2, or DAP-kinase knockout mice) protected retinal ganglion cells from glutamate toxicity but not from the toxicity of NMDA. The results suggest that glutamate-induced neuronal toxicity involves multiple glutamate receptors, the types and relative contributions of which, vary among strains. We suggest that a multifactorial protection, based on an immune mechanism independent of the specific pathway through which glutamate exerts its toxicity, is likely to be a safer, more comprehensive, and hence more effective strategy for neuroprotection. It might suggest that, because of individual differences, the pharmacological use of NMDA-antagonist for neuroprotective purposes might have an adverse effect, even if the affinity is low.

Introduction

The amino acid glutamate is the principal excitatory neurotransmitter in the CNS, where it participates in learning and memory processes. Glutamate activates numerous receptors, each with its own specificity to a particular set of ligands (Nakanishi, 1992). Under normal conditions, extracellular glutamate is maintained at safe physiological concentrations by a number of buffering mechanisms, including uptake of glutamate by glial cells and its conversion by glutamine synthetase or glutamate decarboxylase to the nontoxic amino acid glutamine (Bezzi *et al.*, 1999; Rauen *et al.*, 1999). This procedure replenishes the neurotransmitter pool, while avoiding glutamate neurotoxicity.

Excitotoxicity, caused by an increase in extracellular glutamate, appears to be acutely involved in status epilepticus (Fountain, 2000), as well as in neurodegenerative processes following cerebral ischemia (Dirnagl *et al.*, 1999; Lipton, 1999), traumatic brain injuries, or axonal injuries (Alessandri & Bullock, 1998). It may also promote chronic neurodegeneration (Meldrum, 2000) in amyotrophic lateral sclerosis (Shaw & Ince, 1997), Huntington's chorea (Kieburtz, 1999), glaucoma (Vorwerk *et al.*, 1999) and other CNS disorders. An example of a neurodegenerative disease associated with a local increase in glutamate is the optic nerve neuropathy seen in glaucoma patients (Dreyer, 1998). An abnormal increase in glutamate concentration in the eyes of these patients is thought to be responsible, at least in part, for the ongoing death of retinal ganglion cells (RGCs) despite therapeutic normalization of intraocular pressure.

The neurotoxic effect of glutamate is widely thought to be exerted predominantly by the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor, known to be expressed only on neurons. In animal models of cerebral ischemia (Palmer *et al.*, 1999; Williams *et al.*, 2000), traumatic brain injury (Dempsey *et al.*, 2000), and axonal injury (Yoles *et al.*, 1997; Li & Tator, 1999; Gaviria *et al.*, 2000), antagonists of the NMDA receptor reduce damage propagation. In humans, these agents fail to display a beneficial effect (Mikkelsen *et al.*, 2000). Moreover, they have psychotropic and other side-effects (Krystal *et al.*, 1994; Olney, 1994; Muir & Lees, 1995), making them less suitable for therapeutic purposes. These and other observations raised some new possibilities regarding glutamate toxicity, among them: (i) that the toxicity exerted by an excess of glutamate is mediated by more than one type of glutamate receptor on the neurons; (ii) that glutamate toxicity activates glutamate receptors not only on neurons but also on non-neuronal and non-neural cells, such as resident microglia (Hirayama & Kuriyama, 2001; Rogove & Tsirka, 1998; Watanabe *et al.*, 2000), invading macrophages, and T cells (Rimaniol *et al.*, 2000; Storto *et al.*, 2000); and (iii) that, as

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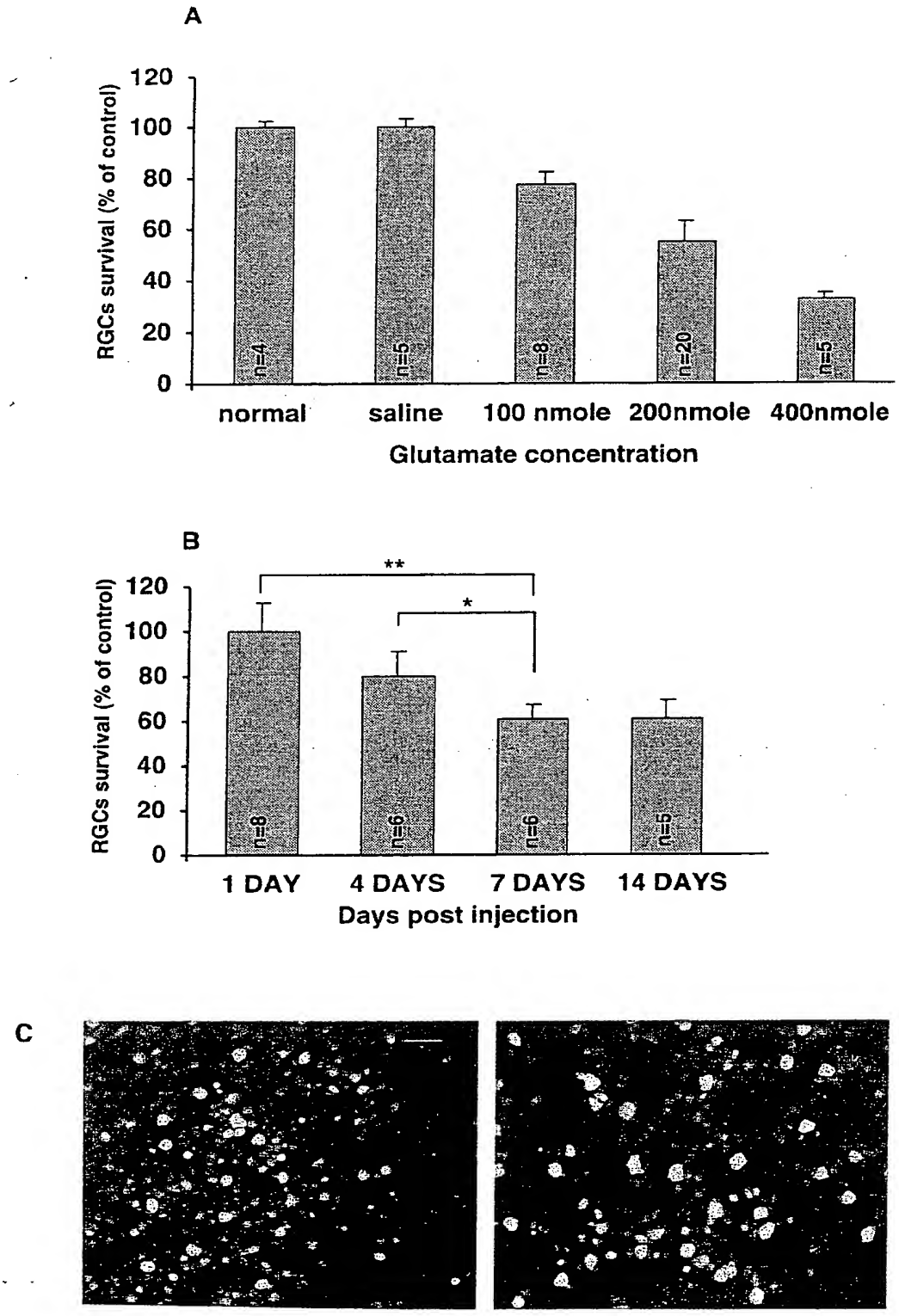

FIG. 1. Dose-dependent and time-dependent retinal ganglion cell death induced by intravitreal glutamate injection in C57BL/6 J mice. (A) Mice received a single injection of glutamate at different dosages (100, 200 or 400 nmol), and surviving RGCs were counted 7 days later. (B) Mice received a single injection of glutamate (200 nmol) and surviving RGCs were counted at different times after the injection ($P < 0.02$, 4 d vs. 7 d; $P < 0.002$, 1 d vs. 7 d. Student's *t*-test). For each treatment, the mean number of surviving RGCs in the injected eyes was expressed as a percentage of the mean number of cells in the contralateral noninjected eyes. Bars represent the percentage survival (mean ± SEM) in each treatment. (C) Photomicrographs of normal retina and glutamate-injected retina, stereotactically labelled with FluoroGold, both excised 7 days after the insult. Calibration, 60 μm.

reviously suggested by our group (Kipnis *et al.*, 2001; Schori *et al.*, 2001a, 2001b), glutamate-induced death signals may differ among individuals, and that these differences might be related, at least in some strains, to T cell-mediated protective mechanism activated by glutamate (Kipnis *et al.*, 2001). Other studies have also referred to the possible influence of genetic background on the consequences of CNS insults (Olsson *et al.*, 2000; Lundberg *et al.*, 2001; Xu *et al.*, 2001).

Little is known about the glutamate receptors or the mechanisms that participate in T cell-mediated protective effects, resistance to cytotoxicity, or systemic neuroprotection from cytotoxicity. In an attempt to gain an insight into the nature of the glutamate receptors that participate in cytotoxicity and to elucidate the T cell-mediated mechanisms of protection from glutamate toxicity, we examined RGC survival after optic nerve injury in a variety of mouse strains differing in their ability to manifest protective T cell mechanisms; in mice devoid of mature T cells; in genetically manipulated mice overexpressing bcl-2 and in mice lacking the death-associated protein kinase [DAP-k knockout mice]. We also examined the effects of pharmacological intervention with the NMDA antagonist, MK-801, or the α_2-adrenoreceptor agonist, brimonidine. The latter is known to up-regulate the neuronal survival factor, basic fibroblast growth factor (bFGF), and to affect apoptotic pathways by inducing *bcl-2* and *bcl-xl* (Enkvist *et al.*, 1996; Wen *et al.*, 1996; Peng *et al.*, 1998; Yoles *et al.*, 1999).

Materials and methods

Animals

Male C57BL/6 J wild-type and male Balb/c wild-type and nude mice, all aged 8–13 weeks, were supplied by the Animal Breeding Center of the Weizmann Institute of Science and were housed in a light- and temperature-controlled room. DAP-k knockout mice were produced at the Weizmann Institute of Science. The bcl-2 mice were the generous gift of Professor O. Bernard (University of Melbourne, Australia). All mice were handled according to the ARVO resolution on the use of animals in research.

Glutamate-induced and NMDA-induced toxicity

C57BL/6 J mice were anaesthetized by intraperitoneal (i.p.) injection of ketamine (80 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (16 mg/kg; Vitamed, Israel). Their right eyes were punctured with a 27-gauge needle in the upper part of the sclera and a Hamilton syringe with a 30-gauge needle was inserted as far as the vitreal body. Each mouse was injected with a total volume of 1 μL saline containing either L-glutamate (100, 200, or 400 nmol; Sigma, St. Louis, MO) or NMDA (50, 75, or 150 nmol; RBI, Boston, MA). Mice in the control group were injected with saline only. Assuming a vitreal volume of 10 μL, the final concentrations were approximately 10–40 mM for glutamate and 5–15 mM for NMDA.

Brimonidine treatment

In one group of mice, the α_2-adrenoreceptor agonist, brimonidine (0.1 mg/kg; Allergen, Irvine, CA), dissolved in saline, was injected i.p. immediately after intraocular injection of glutamate or NMDA. Mice in the control group received i.p. injections of saline only.

Treatment with receptor antagonist

Mice were intravitreally or intraperitoneally injected with the NMDA-receptor antagonist, MK-801 (RBI, Boston, MA), or intravitreally with the amino-3-hydroxy-5-methy-4-isoxazolepropionic acid (AMPA/KA)-receptor antagonist, NBQX (Sigma, St. Louis, MO).

Labelling of retinal ganglion cells

Mice were anaesthetized as described above and placed in a stereotactic device. The skull was exposed and the bregma identified and marked. The site selected for injection was in the superior colliculus, 2.92 mm posterior to the bregma, 0.5 mm lateral to the midline, and at a depth of 2 mm from the brain surface (Franklin & Paxinos, 1997). A window was drilled in the scalp above the designated coordinates in the right and left hemispheres. The neurotracer dye FluoroGold (5% solution in saline; Fluorochrome, Denver, CO), was stereotactically applied (1 μL, at a rate of 0.5 μL/min in each hemisphere) using a Hamilton syringe, and the skin over the wound was sutured. After 72 h the mice were killed with a lethal dose of pentobarbitone (170 mg/kg), their eyes were enucleated, and each retina was detached from the eyes and prepared as flattened whole mounts in 4% paraformaldehyde in phosphate-buffered saline.

Assessment of retinal ganglion cell survival

Retinas were examined for labelled RGCs by fluorescence microscopy. Labeled cells from four to six fields of identical size (0.076 mm^2) were counted. The selected fields were located at approximately the same distance from the optic disk (0.3 mm) to counteract variations in RGC density as a function of distance from the optic disk. Cells were counted under the fluorescence microscope (magnification × 800) by observers blinded to the treatment received by the mice. The average number of RGCs per field was calculated for each retina. The number of RGCs in the contralateral (uninjured) eye was also counted and served as an internal control.

Results

Glutamate-induced toxicity in retinal ganglion cells as a function of dose and time

To determine the relationship between glutamate dosage and the toxicity induced in mouse RGCs, we injected a single dose of glutamate (100, 200, or 400 nmol) into the vitreous of the right eyes in C57BL/6 J mice, a strain known to be susceptible to glutamate toxicity (Kipnis *et al.*, 2001; Schori *et al.*, 2001b). Mice of the same strain were injected with saline only (0.9% NaCl) and served as a control. Seven days later, surviving RGCs in the injected eyes were counted and expressed as a mean percentage of the numbers of RGCs counted in the noninjected contralateral eyes. The mean number of surviving RGCs decreased as the dosage of injected glutamate increased. The mean number of surviving RGCs in the saline-injected eyes did not differ from that in the noninjected eyes, indicating that RGC survival was not affected by the injection procedure itself (Fig. 1A). Mean RGC survival, expressed hereafter (unless otherwise indicated) as a percentage of the number of RGCs in normal retinae (± SEM), was 99.97 ± 3.21 in the saline-injected group, and 77.6 ± 5.03, 55.4 ± 7.51, and 32.96 ± 2.38 in the groups injected with 100, 200, and 400 nmol glutamate, respectively.

To determine the kinetics of glutamate cytotoxicity, we examined RGC death at various times after a single intravitreal injection of 200 nmol glutamate. Death of RGCs within the first 24 h after the injection was not significant, but 20% of the RGCs died by day 4 and 40–50% by day 7 (Fig. 1B). Mean RGC survival following injection of 200 nmol glutamate was 99.6 ± 6.67, 68.3 ± 12.2, 61.2 ± 2.6, and 60.6 ± 3.6 after 1, 4, 7 and 14 days, respectively. Figure 1C

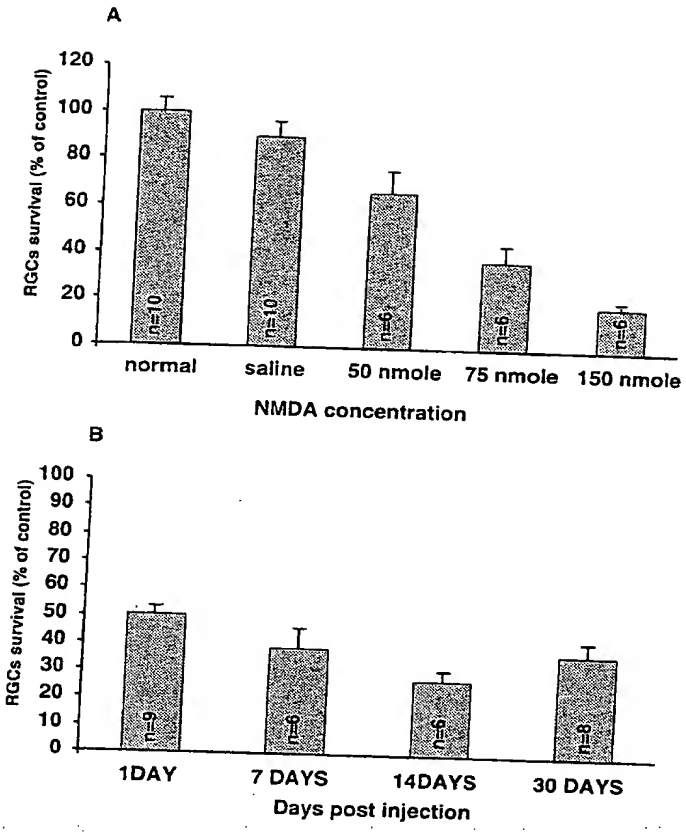

FIG. 2. Dose-dependent and time-dependent retinal ganglion cell death induced by intravitreal injection of NMDA in C57BL/6 J mice. (A) Mice received a single injection of NMDA at different dosages (50, 75 or 150 nmol) or saline, and surviving RGCs were counted 7 days later. (B) Mice received a single injection of NMDA (75 nmol), and surviving RGCs were counted at different times after the injection. For each treatment, the mean number of surviving RGCs in the injected eyes was expressed as a percentage of the mean number of RGCs in the contralateral noninjected eyes. Bars show the percentage survival (mean + SEM) for each treatment.

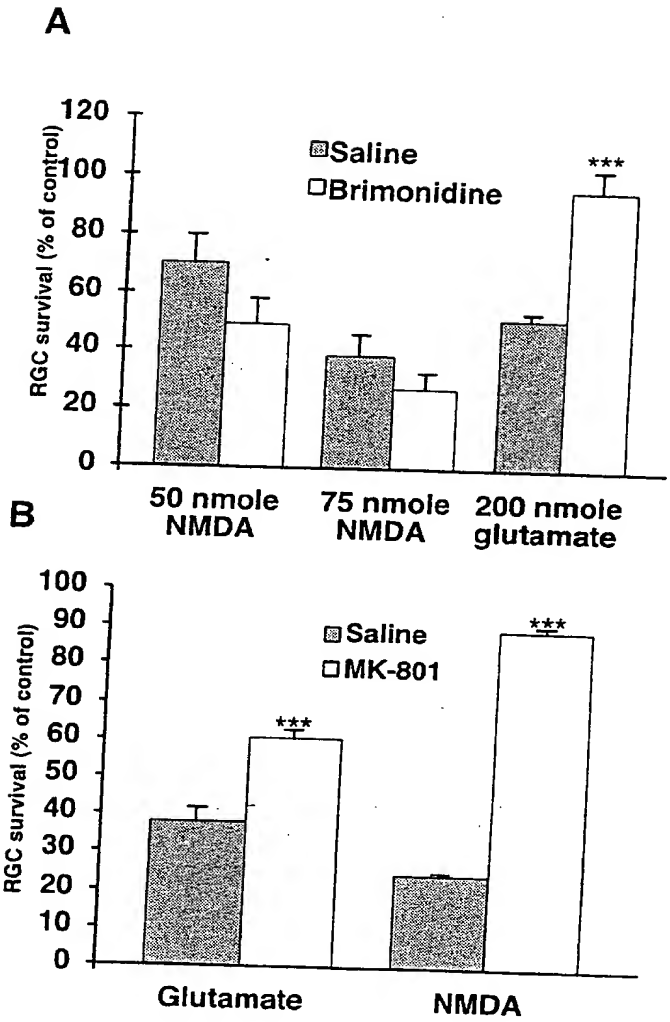

FIG. 3. Protection from glutamate-induced or NMDA-induced toxicity is obtained through different mechanisms. (A) Neuroprotective effect of brimonidine on the toxicity induced by glutamate or NMDA in C57BL/6 J mice. After a single intravitreal injection of glutamate (200 nmol) or NMDA (50 or 75 nmol), mice were injected i.p. with brimonidine (0.1 mg/kg). Mice injected intravitreally or i.p. with saline served as control. Surviving RGCs were counted 7 days later. (B) Protective effect of MK-801 after glutamate or NMDA injection in C57BL/6 J mice. Mice exposed to NMDA (75 nmol) or to glutamate (200 nmol) were injected i.p. with 0.2 mL of MK-801 (1 mg/kg) or 0.2 mL of saline (controls). Surviving RGCs were counted 7 days later. Bars show mean values + SEM of RGC survival in the different groups, expressed as a percentage of RGC survival in the saline-treated control. $P < 10^{-4}$, glutamate vs. brimonidine + glutamate; $P < 10^{-4}$, glutamate vs. MK-801 + glutamate; $P < 10^{-5}$ NMDA vs. MK-801 + NMDA, Student's *t*-test.

shows photomicrographs of normal retina and glutamate-injected retina, both excised 7 days after the insult.

Is glutamate toxicity in RGCs mediated by the NMDA receptor?

Using C57BL/6 J mice, we first examined whether NMDA can mimic the effect of glutamate on RGC death, and in particular its effect on the kinetics of the death process. Accordingly, we sought to determine the concentration of injected NMDA that yields a significant loss of RGCs comparable to the death caused by glutamate. Mice injected with saline served as a control. A dose–response curve showed that death comparable to the death caused by 200 nmol of glutamate is caused by injection of 75 nmol of NMDA. RGC survival was 91 ± 6.1 in the saline injected group and 66.7 ± 9.3, 38 ± 7 and 19 ± 2 in the groups injected with 50, 75, and 150 nmol NMDA, respectively (Fig. 2A). Counting of the surviving RGCs at different times after the administration of 75 nmol of NMDA showed that death induced by NMDA occurs more rapidly than glutamate-induced death. After exposure to NMDA, almost all RGC death occurred within 24 h. In addition, survival of RGCs measured 1 day after NMDA injection did not differ significantly from that measured at any subsequent time. Mean RGC survival was 50.5 ± 2.9, 39 ± 7.2, 30 ± 4.1 and 38 ± 4.04 on days 1, 7, 14 and 30, respectively (Fig. 2B).

To determine whether the observed differences in kinetics reflect different mechanisms of death, we compared the effects of pharmacological or genetic intervention on NMDA-induced and glutamate-induced death, using NMDA antagonists or a neuroprotective agent unrelated to NMDA. For the latter, we chose the α_2-adrenoreceptor agonist, brimonidine, which indirectly protects rat RGCs from death induced by axonal injury (Yoles *et al.*, 1999) by causing up-regulation of survival genes (Enkvist *et al.*, 1996; Wen *et al.*, 1996;

Table 1. Protection (measured by retinal ganglion survival) from toxicity induced by glutamate or by NMDA in genetically manipulated mice

	Bcl-2		DAPk	
	Control	Transgenic	Control	Transgenic
Glutamate	55 ± 6	81 ± 11*	56 ± 4	79 ± 3**
NMDA	30 ± 3	55 ± 5*	40 ± 6	37 ± 3

RGC survival is expressed as a percentage of RGCs in untreated, genetically manipulated mice. Mice overexpressing *bcl-2* or DAP-K knockout mice were treated with glutamate (200 nmol) or NMDA (75 nmol) as described for Figs 1 and 2A. Surviving RGCs were counted 7 days later. $*P < 0.05$, $**P < 0.005$, Student's *t*-test.

Peng *et al.*, 1998). Treatment with brimonidine in this study protected RGCs from glutamate toxicity: mean percentage of RGC survival was 52 ± 2.4 in the retinae treated with glutamate only and 96 ± 6.5 in the retinae treated with glutamate and brimonidine. Brimonidine did not, however, protect RGCs from the toxicity of NMDA (50 nmol): mean percentage of RGC survival was 52 ± 8.7 in the group treated with brimonidine and NMDA, compared to 70 ± 9.8 in the group treated with NMDA only. After injection of 75 nmol of NMDA, the mean percentage of surviving RGCs (± SEM) was 39 ± 7.2 in the group treated with NMDA only and 28 ± 5.7 in the group treated with brimonidine and NMDA (Fig. 3A). In contrast, the specific NMDA-receptor antagonist, MK-801 (1 mg/kg injected i.p), completely blocked NMDA-induced toxicity (percentage RGC survival after NMDA injection was 89 ± 1.46 in the MK-801-treated group and 25 ± 0.57 in the group without MK-801 treatment), but only partially blocked glutamate toxicity (percentage RGC survival after glutamate injection was 60 ± 1.9 in the MK-801-treated group and 38 ± 4.13 in the group without MK-801 treatment) (Fig. 3B). Moreover, in mice overexpressing the survival gene, *bcl-2*, resistance to glutamate toxicity was significantly greater than in matched wild-type mice. The corresponding resistance to NMDA toxicity was only slightly (though still significantly) greater than in the matched wild type (Table 1). In DAP-k knockout mice, resistance to glutamate toxicity was also significantly greater than in their matched wild type, whereas the corresponding resistance to NMDA toxicity was not improved in these mice relative to their controls (Table 1).

Effects of glutamate and glutamate-receptor antagonists on RGC survival

Studies in our laboratory have demonstrated that the extent of recovery from CNS injuries is strain dependent, and that strain-related differences in resistance to the toxic effects of glutamate are correlated with the ability to manifest a T cell-dependent protective response (Kipnis *et al.*, 2001). Here, we examined whether the effects of glutamate-receptor antagonists differ in different mouse strains. First, we compared the effect of glutamate-receptor antagonists in two mouse strains (Balb/c and C57BL/6 J) known to differ in their ability to manifest a T cell-dependent neuroprotective response. The selected antagonists were MK801 (an NMDA-receptor antagonist) and NBQX [an antagonist of the AMPA/KA receptor, known to participate in neuronal death (Ikonomidou *et al.*, 2000)]. The effect of glutamate toxicity was more severe in C57BL/6 J mice than in Balb/c mice (RGC survival was 37% and 61%, respectively), in line with previous findings from our laboratory (Kipnis *et al.*, 2001). In C57BL/6 J mice the glutamate-induced toxicity was partially blocked by both MK801 and NBQX, with resulting RGC survival of 52% and

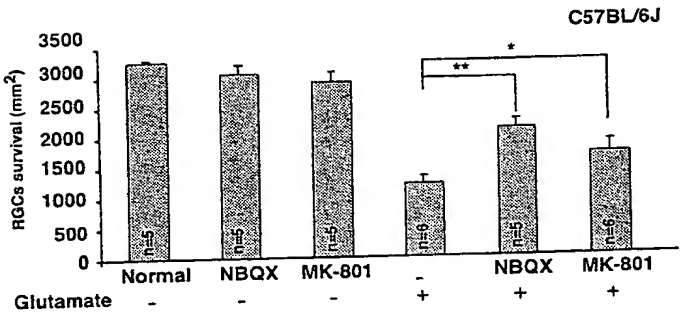

FIG. 4. Protection from glutamate toxicity by pharmacological intervention. Wild-type C57BL/6 J mice were injected intravitreally with MK-801 (0.15 μg/μL), NBQX (0.5 μg/μL), glutamate (200 nmol), MK-801 (0.15 μg/μL) + glutamate (200 nmol), or NBQX (0.5 μg/μL) + glutamate (200 nmol). The number of surviving RGCs per mm² (bars show mean + SEM) was determined 7 days later. $P < 0.03$, glutamate vs. MK-801 + glutamate; $P < 0.005$, NBQX + glutamate vs. glutamate, Student's *t*-test.

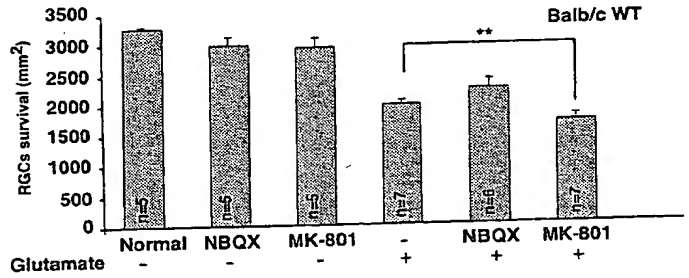

FIG. 5. Protection from glutamate toxicity by pharmacological intervention. Wild-type Balb/c mice were injected intravitreally with MK-801 (0.15 μg/μL), MK-801 (0.15 μg/μL) + glutamate, NBQX (0.5 μg/μL), NBQX (0.5 μg/μL) + glutamate, or glutamate only (200 nmol). The number of surviving RGCs per mm² (bars show mean + SEM) was determined 7 days later. $P < 0.008$, glutamate vs. MK-801 + glutamate, Student's *t*-test.

65%, respectively, compared to 37% in control mice injected with glutamate only. The numbers of surviving RGCs per mm² were 3262 ± 36.5 in normal retinae, 3050 ± 151.2 after treatment with NBQX only, 2914 ± 155.8 after treatment with MK-801 only, 1201 ± 130.6 after treatment with glutamate only, 2113 ± 153.9 after treatment with NBQX + glutamate, and 1694 ± 190.2 after treatment with MK-801 + glutamate (Fig. 4). In Balb/c mice, a strain that spontaneously manifests a T cell-mediated response that protects CNS nerves from the effects of glutamate toxicity, the protective effect of NBQX was small (68% survival compared to 61% in the control), possibly because of an endogenous immune response that partially counteracts the glutamate toxicity. Treatment with MK801, however, not only failed to improve the outcome after exposure to glutamate toxicity, but even exacerbated the toxic effect. This finding is in line with previous reports that the presence of the NMDA receptor is essential for the operation of survival genes. The numbers of surviving RGCs (± SEM) per mm² were 3262 ± 36.5 in normal retinae, 2993 ± 142.7 after treatment with NBQX only, 2933 ± 159.1 after treatment with MK-801 only, 1979 ± 66.07 after treatment with glutamate only, 2228 ± 154.5 after treatment with NBQX + glutamate and 1682 ± 97.1 after treatment with MK-801 + glutamate (Fig. 5). These findings show that glutamate toxicity occurs not only via the NMDA receptor but also partially via the AMPA/KA receptor. Moreover, these results might suggest that the spontaneous T cell-mediated protective mechanism in a mouse strain

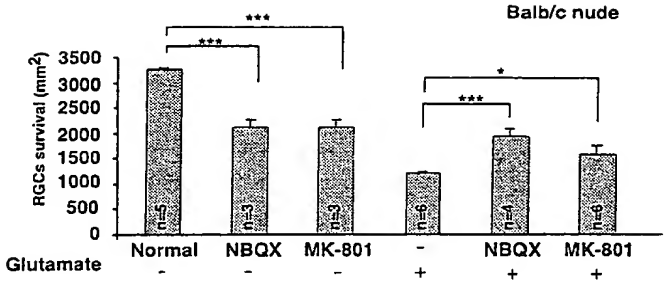

FIG. 6. Balb/c nude mice were injected intravitreally with MK-801 (0.3 μg/μL), NBQX (1 μg/μL) or glutamate (200 nmol). The number of surviving RGCs per mm^2 (bars show mean + SEM) was determined 7 days later. Note that the difference between nude mice injected with glutamate and nude mice treated with MK-801 was significant only according to a one-tailed Student's *t*-test ($P < 0.045$). ***$P < 10^{-3}$, normal vs. NBQX; $P < 10^{-5}$, normal vs. MK-801; $P < 10^{-5}$, glutamate vs. NBQX + glutamate, Student's *t*-test.

that is relatively resistant to glutamate toxicity (Balb/c) is associated with NMDA receptor. It should be borne in mind that glutamate also activates a nonglutamate receptor mechanism, which facilitates delayed neuronal death (Castellano *et al.*, 2001; Grima *et al.*, 2001; Stewart *et al.*, 2002).

To determine whether the T cell-associated protection observed in the Balb/c mice indeed requires the participation of the NMDA receptor, we examined the effects of the two glutamate-receptor antagonists in Balb/c mice devoid of mature T cells. In the absence of mature T cells, the glutamate-induced loss of RGCs in the nude mice was, as expected, greater than in the wild type (RGC survival of 37% compared to 67% in wild type) and was similar to that seen in C57BL/6 J mice (Fig. 4). Moreover, in the absence of mature T cells (i.e., in nude Balb/c mice), MK801 injection had a protective effect on the RGCs (survival of 48% compared to 37% after saline injection). NBQX was also protective in these mice. Thus, the numbers of surviving RGCs were 3262 ± 36.5 in normal retinae, 2100 ± 166.5 after treatment with NBQX only, 2106 ± 145.2 after treatment with MK-801 only, 1205 ± 45.2 after treatment with glutamate only, 1936 ± 156.3 after treatment with NBQX + glutamate and 1559 ± 188.8 after treatment with MK-801 + glutamate (Fig. 6). Treatment with the antagonist alone also apparently leads to some neuronal death, possibly because the antagonist interferes with the maintenance of physiological glutamate levels, which are normally buffered by microglial cells via the glutamate receptors that exist on them. This suggestion is supported by the fact that higher doses of the antagonist increased the amount of neuronal death. These results are similar to those obtained in C57BL/6 J mice (which lack an endogenous beneficial T cell response), suggesting that in the absence of a potent T cell-mediated response, both glutamate receptors have negative effects on neuronal survival and, therefore, their antagonists are protective. These findings further support the suggestion that RGC death induced via the NMDA receptor is counteracted by a T cell-mediated protective response.

Discussion

The results of this study demonstrate that glutamate-induced toxicity in mice is strain dependent, and that the strain dictates not only the extent of glutamate toxicity but also its mechanism of induction. Glutamate, when in excess of physiological concentrations, activates several mechanisms, both local and systemic, aimed at self-protection from its toxic effects. The ability or inability of an individual or strain to activate such mechanisms determines, at least in part, neuronal survival after exposure to glutamate.

We suggest that following a glutamate stress signal, the T cell-dependent arm of the immune system is alerted and activated. The function of the ensuing immune response is to counteract the glutamate toxicity, possibly by activating resident microglia (Butovsky *et al.*, 2001; Schwartz & Kipnis, 2001). Preliminary results suggest that the T cells activate both microglia and astrocytes in a way that increases their buffering capacity. Individuals equipped with this protective T cell-mediated mechanism can tolerate higher doses of glutamate than those lacking such protection. Balb/c mice inherently possess this protective mechanism, but C57BL/6 J mice do not (Kipnis *et al.*, 2001). Because of this lack, glutamate has a more toxic effect in the latter strain of mice (both nude and wild-type) than in Balb/c mice (Kipnis *et al.*, 2001; Schori *et al.*, 2001b) The possibility that T cells can directly respond to glutamate cannot be ruled out, as T cells possess glutamate receptors (Storto *et al.*, 2000).

The AMPA/KA receptor has been shown to mediate neuronal death caused by glutamate toxicity (Ikonomidou *et al.*, 2000; Werner *et al.*, 2000). In addition, the AMPA/KA receptor antagonist is more effective than the NMDA antagonist, MK-801, in protecting RGCs after optic nerve crush injury (Schuettauf *et al.*, 2000). In the present study, treatment with the AMPA/KA-receptor antagonist, NBQX, significantly reduced the extent of glutamate-induced death in all examined mice. In contrast, the NMDA-receptor antagonist, MK801, effectively reduced glutamate-induced neuronal death only in mice with an inherent inability to manifest a protective T cell response [i.e., C57BL/6 J mice known to be highly susceptible to both glutamate toxicity and CNS autoimmune disease induction (Kipnis *et al.*, 2001)], or in mice devoid of mature T cells (nude mice). In Balb/c mice (known to spontaneously manifest a T cell-mediated protective immunity in response to glutamate challenge), treatment with an NMDA-receptor antagonist not only failed to improve the neuronal outcome, but even exacerbated the toxic effect, i.e., fewer RGCs survived in its presence. This observation is in line with reports suggesting that in Wistar rats (resistant to autoimmune disease development) undergoing progressive neurodegeneration or subjected to traumatic brain injury, neuronal losses are increased by NMDA antagonists but reduced by AMPA/KA antagonists (Ikonomidou *et al.*, 2000). It has been suggested that NMDA antagonists, in addition to blocking neuronal activity, might reduce neuronally induced inhibition of microglial expression of MHC-II (Neumann *et al.*, 1996). It is, therefore, possible that glutamate interacts first with the NMDA receptor expressed on neurons, and that this might in turn lead to the activation of microglia, which consequently express MHC-II. The activated microglia then interact directly with glutamate via a non-NMDA receptor (Noda *et al.*, 1999; Noda *et al.*, 2000; Nakajima *et al.*, 2001). These results argue in favour of limited participation of the NMDA receptor in glutamate-induced toxicity. Alternatively, the mechanism and kinetics through which NMDA induces toxicity might allow limited intervention for protection by survival genes or by blocking death-associated genes (Pelled *et al.*, 2002; Cohen & Kimchi, 2001; Raveh *et al.*, 2001). The difference between NMDA toxicity and glutamate-induced toxicity was further manifested by the resistance to these toxins shown by RGCs in mice overexpressing Bcl-2, or mice deficient in DAPk. Thus, compared to matched wild type controls, neither of the transgenic strains showed improved recovery after NMDA injection, whereas recovery was better in both strains after glutamate injection. In addition, our findings might suggest that protective T cells engage in cross-talk with the MHC-II-

expressing microglia, with or without direct exposure to toxic concentrations of glutamate, thereby enhancing their ability to efficiently buffer the potentially harmful conditions that glutamate creates. This proposal is in line with recent unpublished studies in our laboratory showing that T cells can rescue microglia from glutamate toxicity and induce their transformation into antigen-presenting cells. Such an interpretation is consistent with (i) the protection by AMPA/KA and the exacerbating effect of MK801 observed in Balb/c mice and (ii) the protective effects of both AMPA/KA and MK801 observed in nude Balb/c and wild-type C57BL/6 J mice. In these latter mice the T cell-mediated response is lacking, resulting in a defective ability to modulate the toxic effect of glutamate, thus, NMDA-receptor activation leads to death rather than protection.

Our results may explain partially the puzzling observation that NMDA antagonists, which successfully counteracted glutamate-induced toxicity in preclinical studies, had an adverse effect in clinical studies. Up to now, no attention has been paid to the particular species or strain chosen for studies of glutamate toxicity and neuroprotection. We found differences in glutamate-induced death between mice that are endowed with T cell-mediated spontaneous protective mechanisms and those that are not (Kipnis *et al.*, 2001; Schori *et al.*, 2001b). The latter category includes nude mice of strains that inherently possess such protective mechanisms, as well as both wild-type and nude mice of strains that lack such mechanisms. The results of this study also show that glutamate-induced toxicity, even when mediated by the NMDA receptor, differs from NMDA-induced toxicity, both pharmacologically and genetically. Numerous *in vivo* and *in vitro* models have been developed in the search for compounds that can protect neurons against toxicity induced via the glutamate receptor. In many of these studies it was assumed that glutamate could be used interchangeably with its nonmetabolite agonist, NMDA (Li *et al.*, 1999; Kido *et al.*, 2000). The present study shows, however, that when we seek mechanisms of neuroprotection other than those involving the NMDA receptor, we should bear in mind that the use of these two compounds yields very different results, leading to death via mechanisms that involve different genes and display different kinetics. Thus, for example, most of the RGC death induced in mice by intravitreal injection of NMDA occurred within the first 24 h, unlike the death process induced by glutamate, which was more prolonged. Moreover, whereas NMDA-induced toxicity could be completely blocked by the use of its receptor antagonist, glutamate-induced toxicity was only partially inhibited by NMDA antagonists, and not in all cases. In addition, whereas the α_2-adrenoreceptor agonist, brimonidine, shown previously to slow down secondary degeneration (Yoles *et al.*, 1999), was found in this study to protect neurons against glutamate-induced toxicity, it did not protect them from NMDA-induced toxicity.

The differences between glutamate-induced and NMDA-induced death demonstrated in C57BL/6 J mice (which lack a T cell-dependent protective mechanism) might indicate that glutamate stimulates a self-protective pathway alongside the destructive pathway, and that the final outcome is determined by the balance between the two. In addition, glutamate is efficiently cleared from the extracellular environment of the neurons, making its effective dosage lower than the injected dose, as opposed to NMDA, which is neither metabolized nor cleared. Regardless of the death mechanism induced by NMDA, the present study suggests that the search for neuroprotective agents for the treatment of neurodegenerative diseases associated with optic neuropathies should focus on glutamate, and not NMDA, as the mediator of toxicity.

It is also essential to examine the effects of treatment in different strains, as the pharmacological outcome might vary with the nature of the endogenous repair mechanism. The outcome also depends on the mechanism by which the pharmacological intervention operates, whether by interfering with or supplementing the physiological mechanisms. This is an important principle to bear in mind when designing neuroprotective treatment. One way to circumvent strain-related variations might be to choose a universally safe approach, by boosting or inducing an endogenous protective mechanism that operates in all individuals, rather than using a drug that affects a particular pathway or might have different effects in different individuals (Kipnis *et al.*, 2000; Hauben *et al.*, 2001; Schori *et al.*, 2001a).

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Abbreviation

RGC, retinal ganglion cell.

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